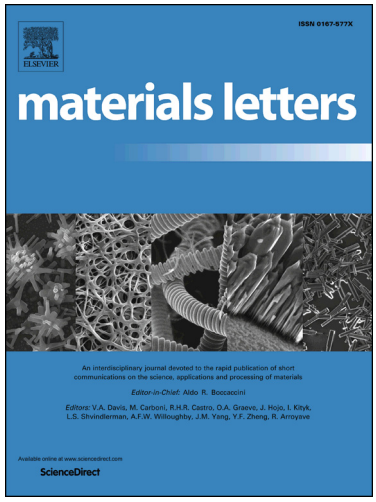

Physico–chemical characterisation and biological evaluation of freeze dried chitosan sponge for wound care

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Physico–chemical characterisation and biological evaluation of freeze dried chitosan sponge for wound care

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Abstract

Haemorrhage is one of the most common cause of mortality if proper action is not taken in a fixed period of time. Over the past three decades, usage of haemostatic materials have been widely introduced for arresting the bleeding. Chitosan is one of the biopolymer which is widely used as a haemostatic agent linked using glutaraldehyde and freeze dried to prepare a spongy haemostat. The process of freeze drying has been optimised in a controlled freeze dryer in such a way that we obtain a sponge having good mechanical strength as well as blood absorbing capacity. The morphology and microstructure of the chitosan sponge was observed by scanning electron microscopy (SEM). The chitosan sponge was sterilised using gamma irradiation and sterility studies were carried out to prove the sterile nature of the material. *In-vitro* cytotoxicity evaluation; *in-vivo* intracutaneous reactivity and Guinea pig maximization test conducted on the

gamma irradiated chitosan sponge proved the safety of the chitosan sponge to be suitable for haemostasis.

Key words: Chitosan, freeze-drying, glutaraldehyde, haemostat, Guinea pig maximization test.

INTRODUCTION

Uncontrolled haemorrhage is the leading preventable cause of death due to accidents and surgical procedures. More than 90% of the world's fatalities on the roads occur in low and middle income countries [1]. In India annually, more than 150 K people die in road accidents. Wide variety of materials such as regenerated cellulose, gelatin, collagen fibres and chitosan are used as haemostatic materials. Nowadays, chitosan based wound dressings are predominant in the wound management system [2]. Chitosan is a natural biopolymer that is derived from chitin, a major component of crustacean outer skeletons [3]. Chitosan is an invaluable material in the field of biomedical engineering and biotechnology with a wide variety of applications that range from skin and vascular grafts to substrates for mammalian cell culture [4]. At the wound site, chitosan will gradually depolymerize to release N-acetyl-β-D-glucosamine, which initiates fibroblast proliferation, and will react with blood and make it clot immediately. It will act as a mechanical barrier on blood till the victim is transported to the hospital. The haemostat on wetting with sterile water turns to a gel which can be wiped off easily without pain. Glutaraldehyde is one of the commonly used cross linker for chitosan. It is less expensive and it reacts with amine groups in chitosan and assist in giving desired texture to the sponge during fabrication under freeze drying conditions.

In the present work we have tried to fabricate and evaluate freeze-dried glutaraldehyde cross linked chitosan sponge for blood haemostasis application [5]. The material was sterilised by

gamma irradiation and the sterility of the materials was confirmed by the sterility test. In this study we have performed the physico-chemical and biological evaluations required for a medical device such as *in vitro* cytotoxicity evaluation and *in vivo* analysis such as intracutaneous reactivity test and Guinea Pig Maximization test (GPMT).

2.1. Experimental

The chitosan sponge was fabricated by lyophilisation in the Lyophilizer (Spinco Biotec, Model-Vertis Genesis, 25L). 2% Chitosan solution was cross linked with gluteraldehyde and poured into Teflon coated aluminium moulds and freeze dried. The chitosan sponge was then packed in an aluminium pouch and sterilised using gamma irradiation at a level of 25 kGy.

2.2. Characterisation of chitosan sponge

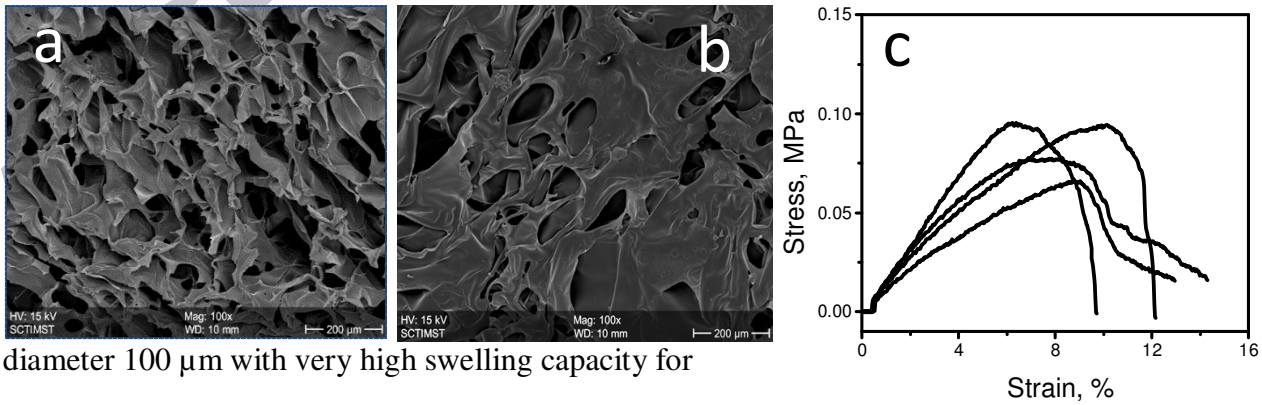
The morphology of the chitosan sponge was examined by scanning electron microscopy (SEM). Molecular weight of the chitosan was found out using Shimadzu LC-10 A HPLC/ GPC system using the column PL aquagel- OH Mixed 8 mm using Dextron standards. DDA was determined by $_1H^1$NMR spectroscopy (supplementary data) [6]. Tensile properties of the sponge were measured using a universal testing machine (Shimadzu-AGX-10kN). Samples in a rectangular shape of dimension 50 mm x 20 mm × 10 mm were tested in the machine at a cross head speed of 50 mm/min and the tensile strength was noted. The experiment was repeated on 5 samples. Blood absorption study was conducted as per British Pharmacopoeia study protocol [7]. Sterility test was conducted based on ISO 11737-2: 2009(E): Sterilization of medical devices-Part 2 and ISO 10993-12:2012(E) [8]. Cytotoxicity was evaluated by ISO 10993-5: 2009 recommended Direct Contact method [9]. Intracutaneous reactivity studies were conducted in accordance with ISO 10993-10: 2010 (E), to evaluate the local response of the chitosan sponge test material extracts following intracutaneous injection into New Zealand white rabbits. The sensitisation

potential of the physiological saline extract of chitosan sponge was evaluated by GPMT in albino guinea pigs (10 animals for test and 5 for control) based on ISO 10993-10: 2010, Biological evaluation of medical devices; Test for irritation and skin sensitization, Clause 7.5: Guinea pig Maximization test [10].

3. RESULTS and DISCUSSION

The molecular weight of the chitosan was determined using gel permeation chromatography and it was found that the molecular weight is around 781 KDa and the molecular weight density was coming to be 13.94.

The inner pore morphology has a role to play in the absorption properties of chitosan sponge [5]. In the present work we have introduced delayed time intervals in the freezing regime for getting a spongy texture with better absorption. Figures 1 a & b shows the scanning electron microscopic images of the chitosan sponge before and after gamma irradiation. In the figure 2a it is seen that the chitosan sponge shows interconnected uniform pores with a diameter of approximately 110 µm. Figure 1b shows that the surface of the sponge is merged after sterilisation but still retains the porosity with a pore diameter of 110 µm. Shao et al, has also reported a porous structure of



diameter 100 µm with very high swelling capacity for the

Fig.1 SEM image of chitosan sponge before (a) and (b) after gamma sterilization at 25KGy (c) stress-strain curve of chitosan haemostat (n = 4)

lyophilised chitosan sponge [11]. The stress strain curve of the chitosan sponge is shown in the fig.1c; it has a break stress of 0.08 N/mm^2 and an elongation at break of 8.3%. This value is comparable to spongostan a gelatin based absorbable haemostat with a break stress of .09N/mm^2 and an elongation at break of 12%.

The *British Pharmacopoeia guidelines* defines dressings with absorption of blood less than 12 g of liquid per 100 cm^2 as with low absorbing efficiency and those with absorption greater than 12 g per 100cm^2 as of high absorbing efficiency (Fig.2) [7]. For the tested chitosan sponge sample absorption was approx. 136 g per 100 cm^2, clearly indicating a high absorption efficiency. Similar studies conducted on two gelatin based haemostats SPONGOSTAN and ABGEL showed an absorption of 128 g/100cm^2 and 107 g/100cm^2 respectively. Further the blood soaked sample was noted for integrity after 24h and it did not torn when grasped by two pairs of forceps. Sterility test performed has revealed no macroscopic evidence of microbial growth in the media with test item extract.

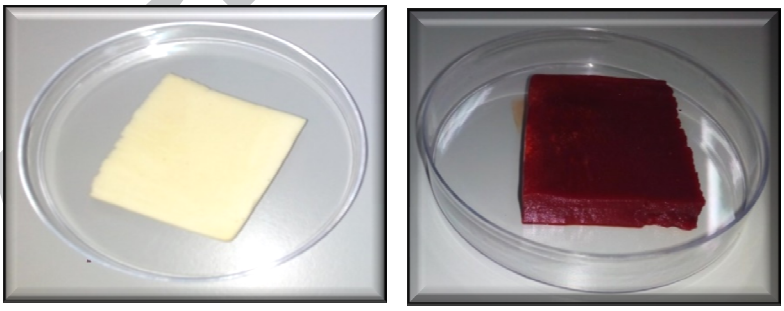


Fig. 2a). Chitosan sponge before and b) after soaking in blood for 30min.

There are several studies in the literature to prove the haemostatic nature of the chitosan sponge, but most of these studies deal with the blood interaction and blood clotting abilities of the sponge [11-13]. In this study we have tried to evaluate the biocompatibility of the haemostatic sponge. Hence the sterilised chitosan sponge was checked for cytotoxicity, intracutaneaous reactivity and GPMT.

4. ***In-vitro cytotoxicity test***

The degree of toxicity was evaluated on the basis of the changes occurring in the cell morphology, their survival rate and ability to proliferate. The investigation showed that after 24h of testing in the L929 cell culture, the cells adhered to the base and maintained regular morphology features with no agglutination, vacuolation, detaching from the base or cell lysis. The cytotoxicity study results indicated that the chitosan sponge sample is non-toxic to the L929 fibroblast cells for the incubated period, proving the material noncytotoxic (Fig.3). Similar studies conducted for chitosan sponge on hepatocytes showed that chitosan sponge is helping in the proliferation of cells [14].

Fig.3. L929 cells after 24h contact with chitosan sponge

4.2. Intracutaneous reactivity test

The intracutaneous reactivity test done as per ISO 10993-10:2010 evaluates the irritation potential of a biomaterial kept in contact with skin. Experiment was done by the intradermal injection of physiological saline (NS) and cotton seed oil extract of the test material in rabbits (test animals= 3, control = 3). The present study indicated that chitosan based hemostats exhibit a nonirritant behavior and no inflammatory response leading to edema or erythema formation. The grading of erythema and edema of the test and control sites of all animals at 24, 48 and 72 h are noted and the difference of the mean reaction scores (erythema/edema) for the test material extracts and the control is zero.

4.3 Guinea Pig maximisation test (GPMT)

GPMT is the preferred test to assess the skin sensitization potential of a medical device or a cosmetic product. The Physiological saline extract of test material and control treated animals did not exhibit any skin sensitization in Guinea pigs, which confirmed that the physiological saline extract of test material is non-sensitive at the simulated laboratory conditions. The GPMT evaluates allergy induced in dermal layer including delayed hypersensitivity by chemicals involved in development stage.

4. Conclusion

The sponges exhibited good absorption capability and biological compatibility (non-cytotoxic and haemostatic properties) besides being flexible and soft; hence it qualifies as a material for homeostasis. The developed chitosan sponges offer very good sorption capacity. Besides the sterilized packed chitosan sponge comply with the test for sterility and non cytotoxicity. The intracutaneous tests in rabbit and Guinea pig maximization tests proved the chitosan sponge is non-irritant and nontoxic. It meets the requirements of the standard practices recommended for haemostatic products with good biocompatibility.

Acknowledgments

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Highlights